Title

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Journal

The journal of advanced prosthodontics, 7(2)

ISSN

2005-7806

Authors

Lee, Dong-Woon
Kim, Jae-Gu
Kim, Meyoung-Kon
et al.

Publication Date

2015-04-01

DOI

10.4047/jap.2015.7.2.138

Peer reviewed

Effect of laser-dimpled titanium surfaces on attachment of epithelial-like cells and fibroblasts

Dong-Woon Lee[1], Jae-Gu Kim[2], Meyoung-Kon Kim[3], Sahar Ansari[4], Alireza Moshaverinia[5], Seong-Ho Choi[6], Jae-Jun Ryu[7]*

[1]Department of Periodontology, Veterans Health Service Medical Center, Seoul, Republic of Korea; Department of Dentistry, Graduate School, Korea University, Seoul, Republic of Korea
[2]Nano-Convergence Mechanical System Research Division, Korea Institute of Machinery and Materials, Daejeon, Republic of Korea
[3]Department of Biochemistry and Molecular Biology, College of Medicine, Korea University, Seoul, Republic of Korea
[4]Division of Periodontology, Ostrow School of Dentistry, University of Southern California, Los Angeles, California, USA
[5]Division of Biomedical Sciences, Ostrow School of Dentistry, University of Southern California, Los Angeles, California, USA
[6]Department of Periodontology, College of Dentistry, Yonsei University, Seoul, Republic of Korea
[7]Department of Prosthodontics, College of Medicine, Korea University, Seoul, Republic of Korea

PURPOSE. The objective of this study was to conduct an *in vitro* comparative evaluation of polished and laser-dimpled titanium (Ti) surfaces to determine whether either surface has an advantage in promoting the attachment of epithelial-like cells and fibroblast to Ti. **MATERIALS AND METHODS.** Forty-eight coin-shaped samples of commercially pure, grade 4 Ti plates were used in this study. These discs were cleaned to a surface roughness (Ra: roughness centerline average) of 180 nm by polishing and were divided into three groups: SM (n=16) had no dimples and served as the control, SM15 (n=16) had 5-μm dimples at 10-μm intervals, and SM30 (n=16) had 5-μm dimples at 25-μm intervals in a 2 × 4 mm^2 area at the center of the disc. Human gingival squamous cell carcinoma cells (YD-38) and human lung fibroblasts (MRC-5) were cultured and used in cell proliferation assays, adhesion assays, immunofluorescent staining of adhesion proteins, and morphological analysis by SEM. The data were analyzed statistically to determine the significance of differences. **RESULTS.** The adhesion strength of epithelial cells was higher on Ti surfaces with 5-μm laser dimples than on polished Ti surfaces, while the adhesion of fibroblasts was not significantly changed by laser treatment of implant surfaces. However, epithelial cells and fibroblasts around the laser dimples appeared larger and showed increased expression of adhesion proteins. **CONCLUSION.** These findings demonstrate that laser dimpling may contribute to improving the peri-implant soft tissue barrier. This study provided helpful information for developing the transmucosal surface of the abutment. ***[J Adv Prosthodont 2015;7:138-45]***

KEY WORDS: Laser; Topography; Attachment; Soft tissue; Dental implant; Epithelial cells

INTRODUCTION

Dental implants comprise an important treatment option for partial or complete edentulism. The functional structure of transmucosal implants entails interfacing with epithelial cells, fibroblasts, and osteoblasts.[1] Since Brånemark coined the term "osseointegration" in the 1960s, the interaction between bone and the Ti implant has been one of the most important and controversial issues in implant dentistry.[2] Recently, unwanted clinical responses such as soft tissue recession and marginal bone resorption have highlighted additional biological and mechanical challenges. Consequently, the soft tissue around the implant has been studied in

Corresponding author:
Jae-Jun Ryu
Department of Prosthodontics, College of Medicine, Korea University
73, Inchon-ro, Seoungbuk-gu, Seoul 136-705, Republic of Korea
Tel. 82 2 920 5425: e-mail, koprosth@unitel.co.kr
Received July 28, 2014 / Last Revision December 3, 2014 / Accepted January 21, 2015

pISSN 2005-7806, eISSN 2005-7814

detail.[3] Additionally, it has been proposed that the stable attachment of soft tissues to the abutment contributes to the resistance of the alveolar bone against bacterial invasion, which can cause peri-implant disease.[4-6] The coronal portion of the implant, beyond the crestal bone level, is generally structured to prevent the accumulation of plaque along the edge of the abutment.[7] In a natural tooth, the connective tissue and epithelium attach to the cemental root surface. The epithelial attachment occurs by means of hemidesmosomes (HDs), and connective tissue attachment results from collagen fibers inserting into the gingival tissues.[8] The coronal portion does not provide such support in an implant. There are limitations to the height of the soft tissue that can surround the implant and thereby resist bacterial invasion of the sulcular epithelium and connective tissue.[9] In animal studies, the structures for epithelial attachment (HDs or basement membranes) are necessarily different from that of a natural tooth.[10] Also, the peri-implant connective tissue attachments resemble scar tissue, consisting of fibroblasts and collagen, with fewer cells than that observed in a healthy periodontal attachment.[11] These studies indicate that the peri-implant soft tissue adhesion should promote the acceptance of implants in the oral environment. To optimize soft tissue attachment, various surface modifications to Ti abutments have been investigated (e.g., fine threads, nano-roughness, and electrolytic polarization).[12-15] Some studies reported that inferior soft tissue cell attachment resulted from a roughened substrate.[3,16,17] However, laser micro-grooves promoted the attachment of connective tissue, which effectively prevented the resorption of peri-implant bone.[18] Although many studies have reported biomechanical modifications, there have been few reports on the biological effects of micro-texturing using the laser dimple technique on the Ti surface.

In this study, we used a laser dimple technique of micro-texturing of dental implant surfaces. It was hypothesized that this process may enhance the adhesion of soft tissue cells to the Ti surface. As this technology entails minimal mechanical modification of surface topography, it may be superior to the rough surfaces used in previous studies, which disturbed soft tissue cell adhesion and accelerated dental plaque accumulation.[3,19] Therefore, the purpose of this study was to conduct an *in vitro* comparative evaluation of polished and laser-dimpled Ti surfaces to determine whether either surface has an advantage in promoting epithelial cell and fibroblast attachment to Ti.

MATERIALS AND METHODS

Forty-eight coin-shaped samples of commercially pure, grade 4 Ti plates (Neobiotech Co., Seoul, Korea) were used in this study. The discs measured 10 mm in diameter and 2 mms thick. They were washed in an ultrasonic bath by using distilled water and stored in 100% ethanol at room temperature before further treatment.

Dimples of 5-μm diameter (Fig. 1) were formed using a 220 fs-pulsed Ti: sapphire laser (wavelength: 800 nm; repetition rate: 100 kHz). The laser beam was focused by a magnification microscope objective IR lens (×20, NA 0.4; Olympus, Tokyo, Japan) by using a laser power of 5 mW and a laser irradiation time of 1 ms to form a dimple. The sample was moved with a velocity of 1 mm/s. Micro-dimpling was performed on a 2 × 4 mm^2 area at the center of the polished Ti discs (Fig. 2). Before irradiation, the sample surface was cleaned to a surface roughness (Ra: roughness centerline average) of 180 nm by polishing with 0.1 μm Alumina powder. The discs were divided into three groups: SM (n = 16) discs were polished Ti discs with no

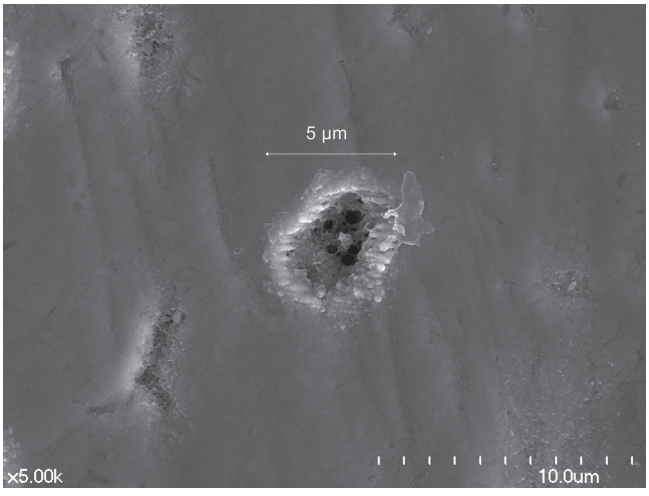

Fig. 1. High magnification (5000×) scanning electron microscopy (SEM) image of a laser-dimpled titanium surface.

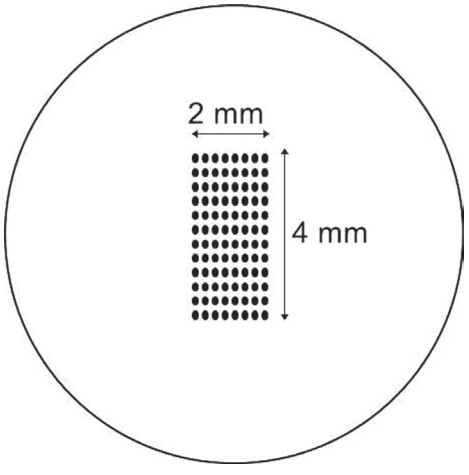

Fig. 2. The 5-μm laser-dimpled surface with an area of 2 × 4 mm^2 at the center of a polished titanium disc.

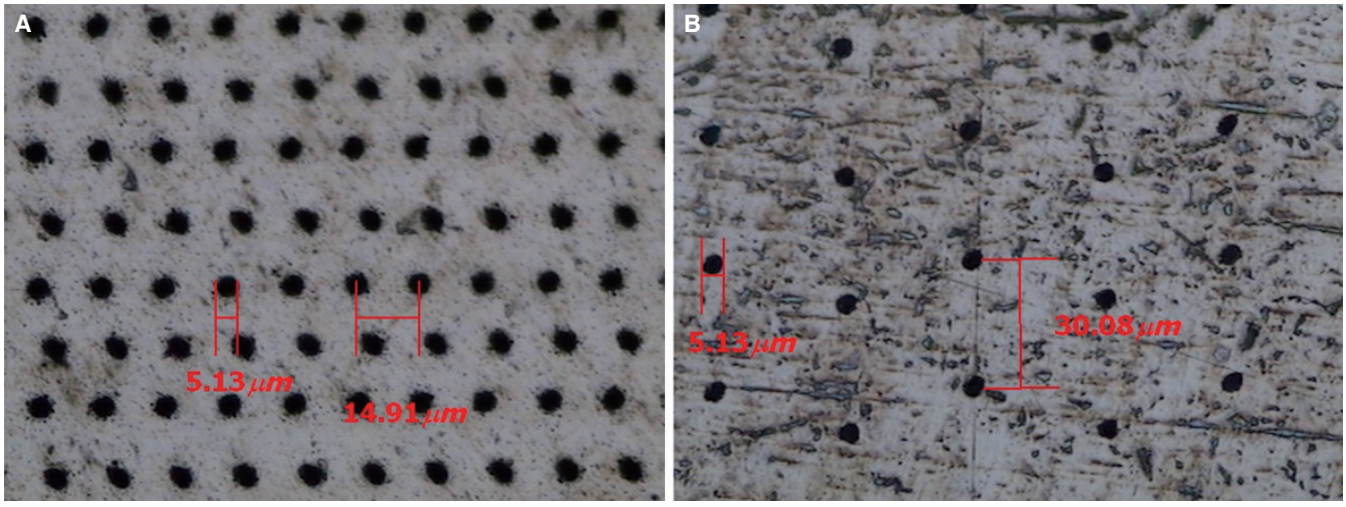

Fig. 3. Light microscopy images of each group (40× magnification). A, SM15: 5-μm dimple and 15-μm center distance. B, SM30: 5-μm dimple and 30-μm center distance.

dimples, which served as controls, SM15 (n = 16) discs had 5-μm dimples at 10-μm intervals (center distance; pitch = 15 μm), and SM30 (n = 16) discs had 5-μm dimples at 25-μm intervals (center distance; pitch = 30 μm) in the polished disc (Fig. 3).

The human lower gingival epithelial squamous carcinoma cell line YD-38 (Korea Cell Line Bank, Seoul, Korea) was cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% L-glutamine, and 2% penicillin/streptomycin solution at 37ºC in a 5% CO_2 humidified incubator. The human fetal lung fibroblast-like cell line MRC-5 (ATCC No. CCL-171) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS, 2% L-glutamine, and 2% penicillin/streptomycin solution at 37ºC in a 5% CO_2 humidified incubator.

WST-8 kit (Dojindo, Kumamoto, Japan) was used to conduct the cell proliferation assay. One Ti disc and 1×10^4 YD-38 or MRC-5 cells were placed in each well of a 24-well plate (Thermo Scientific Nunc, Waltham, MA, USA), and cells were cultured on the Ti disc for 1 or 3 days. These were maintained for 4 hours at 37°C with 1.1 mL of serum-free medium containing 100 μL of WST-8 after removing the culture medium. 100 μL of the supernatant from each well was transferred to a corresponding well with a 96-well plate. The absorbance at 450 nm was recorded in each well by using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). All values of proliferation and adhesion test are expressed as percentage. The size of dimple area (2×4 mm^2) in the entire disc ($5 \times 5 \times 3.14$ mm^2) was assumed as 100 in control disc at day 1.

According to an earlier study, the adhesion assay was conducted for evaluating the adhesion strength of YD-38 and MRC-5 cells.[20] Briefly, cell adhesion strength was measured as follows: 1×10^4 YD-38 or MRC-5 cells were cultured for 1 or 3 days on each type of Ti disc. A rotary shaker (BF-350SK, BioFree, Gyeonggi-do, Korea) agitated the discs three times at 75 rpm for 5 minutes to remove the non-adherent or weakly attached cells. The quantity of adherent cells was measured by the same way, as described in the proliferation assay.

YD-38 and MRC-5 cells were fixed in acetone for 10 min, washed with phosphate-buffered saline (PBS), and blocked with 1% bovine serum albumin at 37ºC. Cells were then fluorescently stained for integrinβ-4, vinculin, and actin filaments, as described below. YD-38 and MRC-5 cells were incubated overnight at 4ºC with mouse anti-integrin β-4 antibody and mouse anti-vinculin antibody (Merck Millipore Co., Billerica, MA, USA), respectively. After washing, cells were incubated with fluorescein isothiocyanate-conjugated (FITC) anti-mouse IgG (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Cells were then washed with PBS and incubated with tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37ºC for actin staining. Subsequently, cells were mounted with 4',6-diamidino-2-phenylindole (DAPI) (VECTOR Laboratories Inc., Burlingame, CA, USA) for nuclear staining. For imaging of the stained cells, a fluorescent microscope (S-4700, HITACHI, Tokyo, Japan) was used.

The morphological characteristics of Ti discs and the cells were analyzed with a scanning electron microscope (SEM, S-4700, HITACHI, Tokyo, Japan).

All statistical analyses were conducted using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA), which calculated the standard deviation (SD) and mean of all data. One-way analysis of variance (ANOVA) with the Duncan's multiple range test was executed to evaluate the differences among groups. A P value <.01 was considered statistically significant.

RESULTS

The number of YD-38 cells increased from day 1 to day 3 by 1.7-fold on SM discs, by 1.9-fold on SM15 discs, and by 2-fold on SM30 discs (Fig. 4). The number of YD-38 cells was the highest when cultured on SM30 discs; however, the differences in cell proliferation among the 3 types of discs were not statistically significant. The proliferation of MRC-5 cells cultured on any of the discs for 3 days showed no significant differences (Fig. 5).

Adhesion assays of YD-38 cells showed no significant differences in adhesion strength among SM, SM15, and SM30 discs on day 1. However, the numbers of adherent YD-38 cells on SM15 and SM30 discs were significantly higher than that on SM discs on day 3 (Fig. 6A). SM15 and SM30 discs had 1.2 times (P<.01, SM15 vs. SM) and 1.5 times (P<.01, SM30 vs. SM) higher number of attached YD-38 cells, respectively, compared to that on SM discs (Fig. 6B). However, the adhesion strength of MRC-5 cell cultures on all discs showed no statistical difference after 1 or 3 days (Fig. 7).

The morphology of YD-38 and MRC-5 cells cultured on different discs was evaluated after 3 days by using SEM. We observed that cells appeared widely spread on the Ti surfaces. In addition, many cellular processes such as filopodia and lamellipodia were commonly observed in both cells on the laser-dimpled areas, in close contact with the underlying Ti surface (Fig. 8). In particular, we found dividing YD-38 cells on the laser-dimpled areas of SM30 discs near the boundary between the dimpled and non-dimpled areas (Fig. 8 - SM30).

To determine the expression levels of adhesion proteins

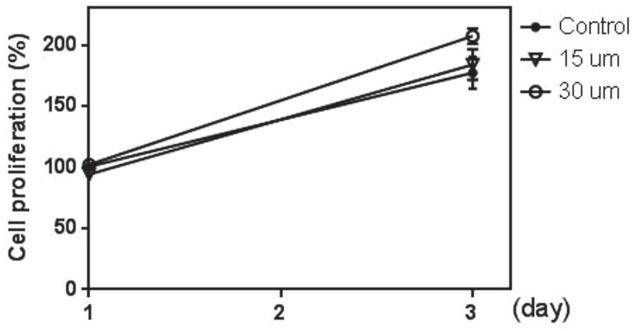

Fig. 4. Proliferation of epithelial cells (YD-38) cultured on titanium discs for 3 days. SM: Smooth surface, served as a control, SM15: 5-μm dimples and 15-μm center distance, and SM30: 5-μm dimples and 30-μm center distance. The value was assumed 100 in control disc at day 1. All values are expressed as percentage.

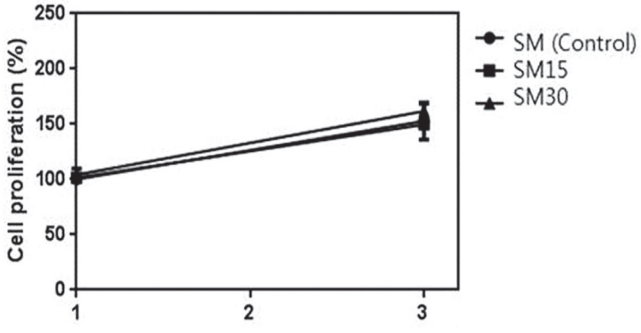

Fig. 5. Proliferation of fibroblast cells (MRC-5) cultured on titanium discs for 3 days. SM: Smooth Surface, served as a control, SM15: 5-μm dimples and 15-μm center distance, and SM30: 5-μm dimples and 30-μm center distance. The value was assumed 100 in control disc at day 1. All values are expressed as percentage.

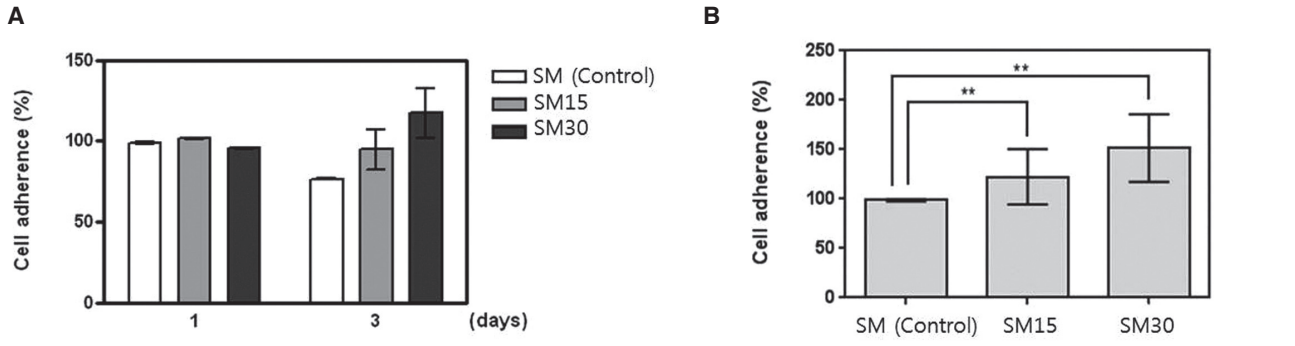

Fig. 6. (A) Adherence of epithelial cells (YD-38) cultured on titanium discs for 1 and 3 days. No significant difference was observed in the adhesion strength between SM, SM15, and SM30 discs on day 1. SM: Smooth surface, served as a control, SM15: 5-μm dimples and 15-μm center distance, and SM30: 5-μm dimples and 30-μm center distance. (B) Cell adherence of YD-38 cells cultured on titanium discs for 3 days. ** P<.01, SM vs. SM15, SM vs. SM30. The value was assumed 100 in control disc at day 1. All values are expressed as percentage.

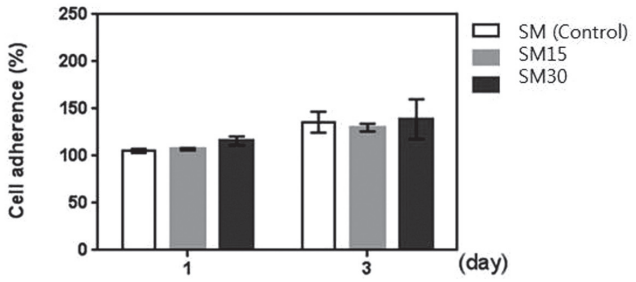

Fig. 7. Adherence of fibroblast cells (MRC-5) cultured on titanium discs for 1 and 3 days. SM: Smooth surface, served as a control, SM15: 5-μm dimples and 15-μm center distance, and SM30: 5-μm dimples and 30-μm center distance. The value was assumed 100 in control disc at day 1. All values are expressed as percentage.

such as actin filaments, integrin-β4, and vinculin in cells cultured on dimpled discs, immunofluorescent staining analysis was performed. As Fig. 9 shows, expression levels of actin filaments and integrin-β4 in YD-38 cells were higher on SM30 discs than on SM discs. Particularly, morphology and cytoskeleton of YD-38 cells appeared more clearly visible on SM30 discs than on SM discs. Actin filaments on SM30 discs extended in a straight line in the nucleus as well as in the cytoplasm. Similar to YD-38 cells in the dimpled areas, the expression levels of actin filaments and vinculin in MRC-5 cells were greater and spread further on SM30 discs than on SM discs (Fig. 10). The expression of vinculin on SM30 discs was clearly distinct in the cytoplasm and nucleus, while on SM discs, such a distinction was not clear.

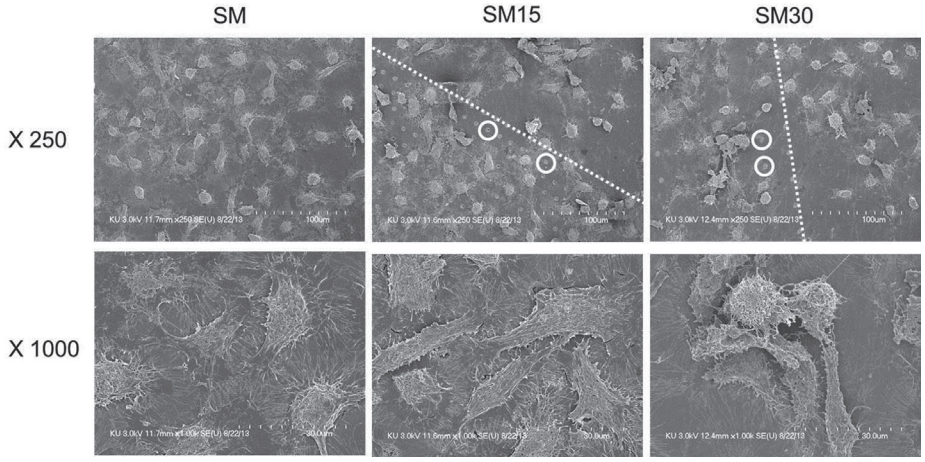

Fig. 8. High magnification (250×, 1000×) scanning electron microscopy (SEM) images of epithelial cells (YD-38) cultured on SM, SM15, and SM30 discs for 3 days. The dotted line represents the border between the dimpled and non-dimpled areas. Two circles among the visible dimples textured on the disc were marked, and imply the dimple zone. SM: Smooth surface, served as a control, SM15: 5-μm dimples and 15-μm center distance, and SM30: 5-μm dimples and 30-μm center distance.

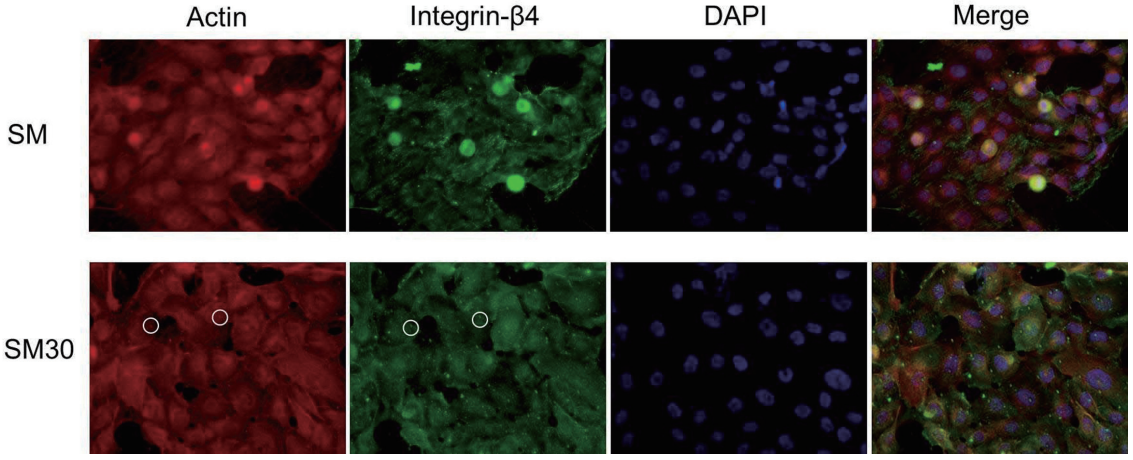

Fig. 9. Immunofluorescence images (400× magnification) showing actin filaments and integrin-β4 in epithelial cells (YD-38) cultured on SM and SM30 discs for 3 days. White circles indicate the laser dimples. SM: Smooth surface, served as a control and SM30: 5-μm dimples and 30-μm center distance on a polished titanium surface.

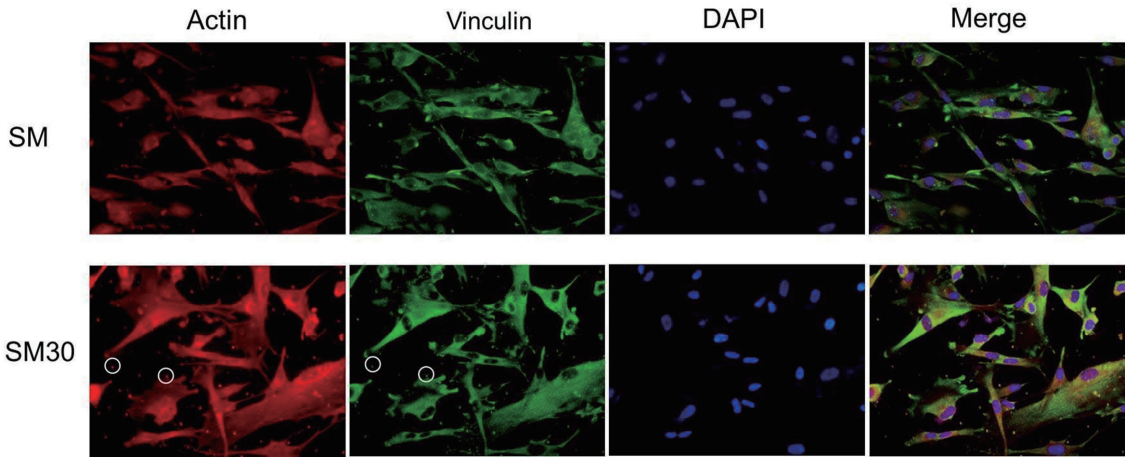

Fig. 10. Immunofluorescence images (400× magnification) showing actin filaments and vinculin in fibroblast cells (MRC-5) cultured on SM and SM30 discs for 3 days. White circles indicate the laser dimples. SM: Smooth surface, served as a control and SM30: 5-μm dimples and 30-μm center distance.

DISCUSSION

Mucosal tissue consists of epithelial and connective tissue components.[6] The epithelial components form the first barrier of the oral environment, while the connective tissue helps to prevent epithelial ingrowth and establish peri-implant sealing.[3,8,21] Notwithstanding a myriad of previous studies, there is no consensus about the optimal surface for effective sealing. Several authors suggested an implant design with a smooth and a hydrophilic neck section that could promote a tight biological sealing by epithelial cells and fibroblasts.[22-24] In this regard, we demonstrated that an altered surface is not significantly different from the existing smooth surface, allowing for similar quantities of plaque accumulation.[25]

This study was designed to examine the effects of 5-μm dimples produced by a laser on a titanium surface. To investigate the effects of laser dimpling, we measured cellular adhesion strength and expression of adhesion molecules in human epithelial cells (YD-38) and human fibroblast cells (MRC-5) cultured on laser-dimpled and control Ti discs. Our data showed that the adhesion strength of YD-38 cells on discs with 5-μm dimples was significantly higher than that of cells cultured on SM (control) discs, even though we did not find any significant differences in cell proliferation among these three types of disc. The results of fluorescence assays showed that the expression of actin and integrin, which are key molecules of cell adhesion processes, was increased in YD-38 cells around the laser dimples, when compared to that observed in cells growing on the control discs. It is well known that the peri-implant epithelium protects the interface between the implant and the bone against bacteria, following implant placement.[26] Moreover, studies have shown that the epithelium attaches to the implant surface mainly through basement membrane HDs. HDs are adhesion plaques in the plasma membranes of epithelial cells that adhere to the extracellular matrix (ECM).[10] HDs are multiprotein complexes, including laminin, integrin, and plectin, among other constituents. The interface between the epithelium and the implant plays a key role in defense and in bonding mechanical closures.[27] The enhancement of epithelial cell attachment, spreading, and HD assembly improves the healing process of soft tissues around the implant.[28] After all, it may be clear that the attachment of epithelial cell has a significance.

Some recent studies indicated that epithelial cells would attach less tightly to rough surfaces.[29,30] These studies also suggested that an engineered surface is advantageous for forming a stable epithelial seal. In this regard, our method might be a positive development because the epithelial cells around the laser dimple design showed an increase on a quantitative scale.

In contrast, 5-μm dimples had no effect on cell proliferation or adhesion strength of MRC-5 cells. This may be due to several reasons. First, fibroblast cells contain high level of fibronectin and fibrin, which generates rapid and secure fibroblast adhesion to the Ti substrate.[19] In other words, the altered surface topography in our study did not affect the proliferation or the attachment of the fibroblast cells because of their superior proliferative capacity. The second reason may be the limitation in the number of dimples because these were not generated on the entire disc. For technical reasons, the dimpled zone was limited to a 2 × 4 mm^2 area at the center of the polished disc. The total number of dimples on each disc was 35,378 (266 × 133) for SM15 discs and 8,778 (133 × 66) for SM30 discs. We

considered these numbers to be enough to estimate potential differences. The third reason may be aging of the Ti surface, causing the surface to become hydrophobic, thereby hindering the attachment of cells.[31] Our study focused on the surface of the abutment in the upper part of the crestal module, which is difficult to maintain in a pristine state, unlike the part below the crestal bone. Therefore, the situation seems to be different from that observed with the adhesion of osteoid cells. In addition, sometimes, the abutment is made to be removable. The last reason may be the size of the dimples. In some Ti surface studies, the orientation of cells was changed depending on groove depth and width.[32,33] Accordingly, the SEM images show that MRC-5 cells covered the dimpled areas. Fluorescent images of adhesion molecules in MRC-5 cells, cultured on discs with 5-μm dimples, showed elevated signals compared to those observed with cells cultured on non-dimpled controls. The cytoskeletal protein vinculin contributes to the mechanical link between the contractile cytoskeleton and the ECM through integrin receptors. In addition, vinculin modulates the dynamics of cell adhesion and is associated with decreased cell motility on two-dimensional ECM substrates.[34,35] In our study, vinculin expression was increased in MRC-5 cells around the laser dimples compared with that in cells cultured on SM (control) discs. This increase in vinculin expression may be related to cell adhesion strength.[3]

CONCLUSION

The results of the current study can be summarized as follows:

1. The adhesion of epithelial cells was higher on titanium surfaces with 5-μm laser dimples than on polished Ti surfaces ($P<.01$).
2. The adhesion of fibroblasts was not significantly changed by laser treatment of implant surfaces.
3. Epithelial cells and fibroblasts around the laser dimples appeared to be larger and showed increased expression of adhesion proteins.

These findings indicate that laser dimpling may promote the quality of the soft tissue seal around dental implants. This study provided helpful information for developing the abutment surface of implants. Nevertheless, optimization through animal and clinical studies is required to demonstrate the potential use of laser-dimpled Ti surfaces in implant dentistry.

ACKNOWLEDGEMENTS

We thank to Prof. Jae-Seok Im for providing the general advice, Prof. Kyung-Lhi Kang for editing English, and Yu-Ri Kim for the technical support.

ORCID

Dong-Woon Lee *http://orcid.org/0000-0002-0796-9100*

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